Isolation and Cultivation of Lyme Disease Spirochetes

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The successful isolation and cultivation of Lyme disease spirochetes traces its lineage to early attempts at cultivating relapsing fever borreliae. Observations on the growth of Lyme disease spirochetes under different *in vitro* conditions may yield important clues to both the metabolic characteristics of these newly discovered organisms and the pathogenesis of Lyme disease.

The isolation and *in vitro* cultivation of hitherto unknown spirochetes first from ixodid ticks [1,2] and then from mammals [3,4] and patients with Lyme disease [5–7] was an advance that was built upon a substantial foundation provided by other investigators. A distant connection exists to the attempts by Noguchi at the Rockefeller Institute to cultivate pathogenic spirochetes. Although the treponeme he eventually propagated was not, in retrospect, *Treponema pallidum* [8,9], it is clear that in his laboratory relapsing fever borreliae could be maintained, at the very least, *in vitro* and probably did multiply for several generations [10]. His medium for borreliae had as its base human ascitic fluid, which to be optimally conducive to borrelial growth had to be free of bile, high in protein content, and capable of producing a disperse fibrin net upon addition of citrated blood [10,11]. These observations may have anticipated the exquisite sensitivity of borreliae to detergents [12] and the need for albumin and a thickening agent such as gelatin in latter-day media formulations [2,13,14].

In 1922 Kligler and Robertson, also at the Rockefeller Institute, extended the research on borrelial cultivation by defining the conditions for maintenance and growth of these spirochetes in derivatives of Noguchi's medium [15]. These investigators found, for instance, that rabbit or horse serum, even when diluted, could substitute for human ascitic fluid and that growth effectively occurred only between pH 7 and 8. To "stabilize" the pH, Kligler and Robertson added peptone water or egg albumin as "buffer." The salutariness of these additives may have been as much from the additional nutrients they supplied as from their buffering capacities. The two workers also identified borreliae as aerobes and noted that the constancy of the carbon dioxide concentration was important.

Although there were many other attempts in the subsequent half century to propagate borreliae continuously, these efforts were, for the most part, based upon the animal sera and protein-rich fluids that had been identified earlier. Felsenfeld in his 1971 review of this subject may have been prescient when he said, regarding borrelial

cultivaton: "There is little hope that any of these methods could be used successfully in routine diagnostic laboratories. Further investigations may lead to the development of more feasible methods" [16]. For it was in the publication year of Felsenfeld's monograph that Kelly reported the breakthrough of successful propagation of *Borrelia hermsii* through many passages [13]. Kelly's medium had some of the elements of previous recipes. In addition, however, it contained an adequate buffer system, other salts, glucose, pyruvate, gelatin, sodium bicarbonate, and what I think may have been the single most critical ingredient, N-acetylglucosamine, a building block of bacterial peptidoglycan.

Although Kelly's medium provided a suitable environment for hundreds of generations of borreliae, Stoenner noted in 1974 that this medium could not support the growth of blood culture inocula containing less than 800 spirochetes [17]. To find a medium that would allow the multiplication of one mouse-virulent borrelia into a large population, Stoenner enriched the basic formulation of Kelly. The supplements that permitted cloning of *B. hermsii* were Yeastolate in concert with CMRL 1066, a tissue culture medium containing amino acids, vitamins, and other growth factors [14].

It was from Dr. Stoenner that I learned the science and art of cultivating borreliae, and it was on November 13, 1981, that another colleague at the Rocky Mountain Laboratories, Dr. Willy Burgdorfer, provided me with a pool of midguts from *Ixodes dammini* ticks collected on Shelter Island, New York, by Dr. Jorge Benach and co-workers. I placed the spirochete-containing tissues in the "fortified Kelly's medium" of Stoenner [14] and ground the tissues. Tenfold dilutions of the suspension were made in this medium, and the capped tubes were incubated at 35°C. On November 18, the following was recorded in the lab notebook: "[tubes] #4, 5, 6 all have spirochetes! Borrelia-like." During the next two weeks I tried to rid the culture of its contaminating gram-positive rod and an actinomycete-like bacterium. I found that the spirochete was resistant to 100 μg and 200 μg per ml of, respectively, nalidixic acid and 5-fluorouracil and that these agents suppressed the growth of the contaminants and allowed indirect immunofluorescence assays to be performed with sera collected by Dr. Edgar Grunwaldt on Shelter Island. Shortly thereafter, I noted that cultures containing the 1-to-10,000 and 1-to-100,000 dilutions also showed growth of the unknown spirochetes and that these cultures were free of bacterial contaminants [1]. This original Shelter Island isolate was cloned from the 1-to-100,000 dilution culture by limiting dilution. One of the clones was designated strain B31 (ATCC 35210; 2).

Attempting to improve the medium and the ease of its production, I made some changes. One of the new formulations was used to isolate from *Ixodes ricinus* ticks of Switzerland a spirochete that was practically identical to the *I. dammini* spirochete [2]. This European spirochete (ATCC 35211) was cloned by limiting dilution in the now-named BSK medium [2].

The latest modification, BSK II, is described in Table 1. This medium differs from the original BSK (BSK I) in the deletion of glutamine from CMRL 1066 and addition of Yeastolate. Although many of the ingredients in BSK II, e.g., citrate, pyruvate, gelatin, CMRL 1066, Yeastolate, and even serum (vide infra) may not be essential for growth of the Lyme disease and ixodid tick spirochetes, we have found in our laboratory that only the complete medium suffices for reliable achievement of spirochete densities of 1–4 × 10^8 per ml, generation times of approximately 11–12 hours, and growth of cultures from inocula of one to two organisms. We also use BSK II

TABLE 1
Formulation of BSK II Medium

1. After detergent cleaning, all glassware is rinsed thoroughly with glass-distilled water and then autoclaved.
2. To 900 ml of glass-distilled water are added 100 ml of 10X concentrate of CMRL 1066 without glutamine (Gibco Laboratories, Grand Island, NY, No. 330-1540).
3. Add to the 1X CMRL 1066 in the following order: 5 g Neopeptone (Difco Laboratories, Detroit, MI); 50 g bovine serum albumin, Fraction V (Miles Laboratories, Elkhart, IN, No. 81-003); 2 g Yeastolate (Difco); 6 g N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES, Sigma Chemical Co., St. Louis, MO); 5 g glucose; 0.7 g sodium citrate; 0.8 g sodium pyruvate; 0.4 g N-acetylglucosamine (Sigma); and 2.2 g sodium bicarbonate.
4. Adjust pH of medium at 20-25°C to 7.6 with 1 N NaOH.
5. Add 200 ml of 7 percent gelatin (Difco) which had been dissolved in boiling water.
6. Sterilize by filtration with air pressure (0.2 μm nitrocellulose, Millipore Corp., Bedford, MA). Store medium at 4°C.
7. Before use, add unheated rabbit serum ("trace hemolyzed"; Pel-Freez Biologicals, Inc., Rogers, AR) to a final concentration of 6 percent.
8. Dispense to glass or polystyrene tubes or bottles. Fill containers to 50 to 90 percent capacity and cap tightly.*
9. Incubate at 34-37°C.

*Hamilton, Montana, is at an altitude of 1,200 m. At lower elevations the containers may need to be filled to a higher level.

for growing virulent strains of some of the relapsing fever borreliae. These latter spirochetes are even less tolerant of subtractions or modifications of the BSK II medium than are the Lyme disease spirochetes, and so, for practical reasons, we continue to use this rather complex concoction.

Undoubtedly, useful simplifications of the medium for Lyme disease spirochete cultivation will be discovered, certainly as more is learned of the metabolism and growth requirements of these organisms. On the other hand, insights into these fundamental characteristics of the spirochetes may come also from observations of what empirically works in a medium and what does not. For example, I found that RPMI 1640, a tissue culture formulation less complex than CMRL 1066, could be substituted for the latter supplement in the cultivation of Lyme disease spirochetes but not that of relapsing fever borreliae. Likewise, Brain Heart Infusion broth with 0.5 percent Casamino acids and a salts solution could serve as a replacement for both CMRL 1066 and Neopeptone in the BSK II formulation and allow satisfactory growth of strain B31. However, this altered medium was unsatisfactory for propagation of *B. hermsii* [Barbour AG: unpublished observations]. Following up the discovery by Rothenberg, Kaplan, and co-workers [Kaplan M: personal communication] that Lyme disease spirochetes could be grown in BSK II medium without serum, I found that was indeed possible with some sources of Fraction V of bovine serum albumin (e.g., Sigma A4503) but not with bovine albumin of higher grade (e.g., Miles Laboratories 81-003) as assessed by the albumin content given in the product's specifications [Barbour AG: unpublished observations]. As in the examples given above, *B. hermsii,* in contrast to Lyme disease spirochetes, grew poorly in the absence of serum.

A few additional observations during the two years my laboratory has been cultivating Lyme disease spirochetes and altering the growth conditions may have rele-

vance to the pathogenesis of erythema chronicum migrans, tick-borne meningo-radiculitis, and Lyme arthritis.

First, strain B31 grew both within and as a "lawn" on a BSK I medium solidified with 0.8 percent agarose. The tubes of medium were incubated in 10 percent carbon dioxide and the plates were incubated in a candle jar [Barbour AG: unpublished observations]. This capability to grow in solid medium may denote a potential for these spirochetes to grow in tissues as well as in body fluids. Indeed, spirochetes have been seen in and recovered from skin biopsies of erythema chronicum migrans lesions [5,7].

Second, the optimum temperature for *in vitro* cultivation of Lyme disease spirochetes is between 30–37°C. At 39°C, growth is slower and long filamentous forms appear. At temperatures of 40°C and above, growth does not occur at all, and spirochetes kept at this temperature do not seem capable of multiplying even when the temperature is lowered [Barbour AG: unpublished observations]. Such temperature maxima of around 40°C may explain the localization of these organisms in the skin, a cooler region of the body. It may also indicate that *in vivo* growth is not possible in some animals with relatively high core temperatures.
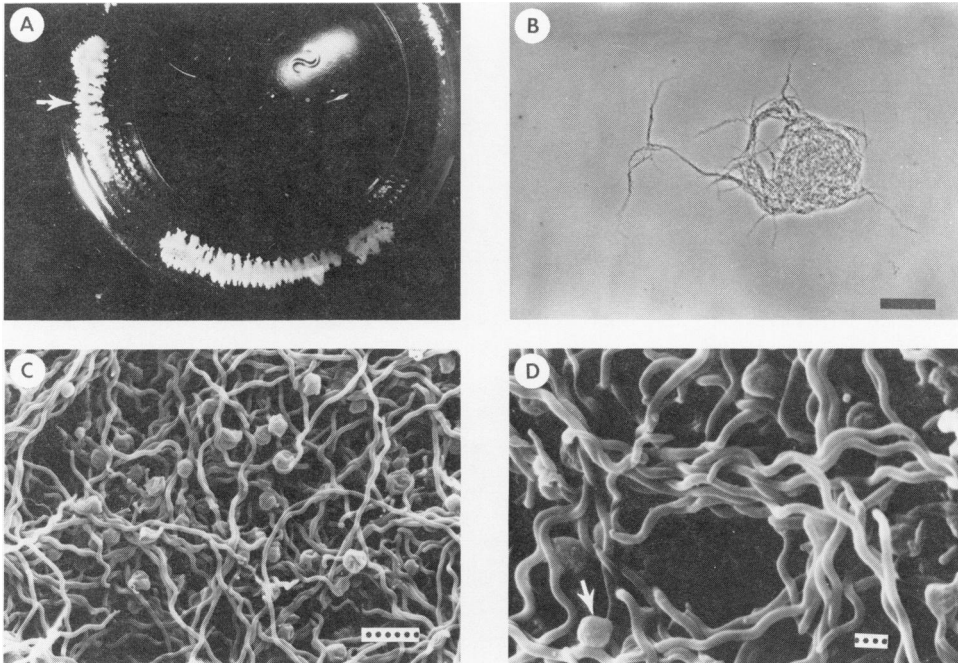

FIG. 1. Aggregation of Lyme disease spirochetes during *in vitro* cultivation.   **A.** Large aggregates of strain HB19 spirochetes (human blood isolate from [5]) at bottom of culture bottle containing BSK II medium. Arrow indicates an aggregate and its characteristic "branches."   **B.** Phase contrast photomicrograph of early passage of the *Ixodes ricinus* spirochete [2]. A small aggregate is shown. Bar, 10 μm. **C.** Scanning electron photomicrograph of aggregate of a field mouse spirochete isolate (strain 50-2; [3]). An aggregate similar to the one shown in frame **A** was carefully removed from the bottom of a culture bottle, fixed in glutaraldehyde, post-fixed in osmium tetroxide, dehydrated in ethanol, critically point-dried in carbon dioxide, and sputtered coated with gold. The figure shows a tangle of spirochetes, many of which have outer membrane blebs. Bar, 1 μm.   **D.** Scanning electron photomicrograph. The same specimen as in frame **C** was prepared as described above. The arrow indicates an outer membrane bleb. Bar, 1 μm.

Finally, I have been intrigued by the tendency of the Lyme disease spirochetes in the initial isolations and through their early subpassages to grow in large aggregates [2]. Examples of those aggregations viewed both macroscopically and microscopically are shown in Fig. 1. Following dispersion of the clumps by vortexing or pipetting, they rapidly reassemble. Many of the strains after several subpassages no longer aggregate and instead swim free in the medium. The apparent "stickiness" of early passage populations may reflect important *in vivo* phenomena. These could include tissue binding in tick and/or mammalian hosts and resistance to phagocytosis through formation of these micro- and macrocolonies.

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